MANUFACTURING AND CHARACTERIZATION OF RESORBABLE PLGA MEMBRANES FOR BIOMEDICAL APPLICATIONS

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Abstract

Porous poly(L-lactide-co-glycolide) (PLGA) membranes were prepared by solvent-casting/porogen leaching method. Poly(ethylene-glycol) (PEG) with two molecular weights was used as a pore former. Mechanical properties of the membranes were analyzed in tensile test. Topography, pore size and surface roughness were characterized by atomic force microscopy on both sites of the membranes. PEG leached out percentage, thickness and wettability were also measured. Osteoblast-like cells were cultured on the membranes for 24 h and 6 days, and morphology, distribution and number of adhered cells as well as secretion of proteins and nitric oxide were measured. The results show that PEG molecular weight affected size and distribution of pores on both surfaces of the membranes. It resulted also in different mechanical characteristics of the membranes. In vitro experiments show that the membranes support adhesion and growth of osteoblast-like cells suggesting their usefulness for guided tissue regeneration (GTR).

Keywords: *PLGA, PEG, porous membrane, phase-separation method, GTR*

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Introduction

Membranes are widely used in medical applications, e.g. for separation and purification purposes, as scaffolds for tissue engineering, in drug delivery systems, artificial organs, diagnostic devices and guided tissue regeneration (GTR) [1-4]. GTR, a biological treatment concept, is aimed to ensure that cells with capacity to regenerate a particular type of lost or diseased tissue are allowed to colonize the defect/wound during healing using a barrier membrane [5]. This procedure was developed particularly for the treatment of dental bone defect to ensure that periodontal ligament cells, bone cells, and cementoblasts selectively repopulate the periodontal wound area and protect the unwanted re-growth of the gingival epithelium and connective tissue [6,7].

Membranes can be prepared by different methods such as electrospinning [8], sintering [2] and freeze-gelation method [9]. Another method that is of great interest because of the simplicity and cost efficiency is phase separation [10,11]. In this method, usually there are two types of polymers present: one of them remains in the end-use and the other is removed as a pore former [12]. Phase separation was used to produce porous poly(L-lactide) membranes with PEG as a porogen by Nakane et al. [13] and Tsuji et al. [14].

PLGA is an interesting synthetic polymer to produce barrier membranes for GTR in periodontology, because of their biodegradability and biocompatibility [15,16]. Moreover its advantage is degradation by hydrolysis resulting in release of non-toxic degradation products, i.e. lactic acid and glycolic acid. These compounds are produced naturally in the human body in different physiological pathways and eliminated by Krebs cycle as water and carbon dioxide, so their toxicity is minimal [17].

PEG is a polymer widely used in several biotechnological and biomedical applications. For example, it prevents protein adsorption, facilitates formation of multi-phase polymeric systems; it is also non-immunogenic and non-antigenic. With its low reactivity and high solubility in water it has attracted much attention to be used as a pore former [13-14,18].

It was shown that depending on the molecular weight and concentration of the porogen, solvent type and weight ratios of the polymers, it is possible to control the microstructure of the resulting membranes [2,4,10]. Owen *et al* [19] studied cell behavior in PLGA/PEG blends, and concluded that these membranes have a great potential to be used in clinic, more specifically in GTR.

The goal of this study was to produce PLGA porous membranes by using PEG with two different molecular weights to obtain specific porous microstructures. The pores were created by leaching out water-soluble PEG from PLGA/PEG blends. A physical and mechanical characterization of the membranes was performed to study their applicability in GTR. Moreover, a biological experiment was conducted in order to provide information about biocompatibility and interaction between osteoblast-like cells and the membranes.

Experimental

Materials

PLGA with 85:15 molar ratio of L-lactide and glycolide and molecular weights M_n=100 kDa and M_w=210 kDa was synthesized in bulk by ring-opening polymerization using low toxic zirconium compound as an initiator [16]. As a pore former, PEG purchased from Aldrich, Germany (molecular weight M_n=1450 Da and M_n=200 Da) was used.

Membrane preparation

PLGA was dissolved in dichloromethane (POCh, Gliwice, Poland) (10% wt/vol.) with magnetic stirring overnight. Then 60 wt% of PEG was added to the PLGA solution and left under stirring during 20 min. After that 7.5 ml of the solution was slip-casted on a Petri dish and dried in air and in vacuum. Next PEG was leached out in ultra-high quality water (UHQ, Elga Purelab, UK) during 4 days, to obtain a porous structure. The resulting membranes were cut into circles (1.4 cm in diameter) and sterilized in ethanol (70%). After, they were washed in phosphate buffer saline (PBS) and UV light-sterilized for 20 min at both sides.

Membrane characterization

Atomic force microscopy (AFM)

The topographical images of both surfaces of the membranes were taken on atomic force microscope (Explorer, Veeco, USA) in contact mode at room temperature. The size of pores and roughness (Ra) of the obtained membranes were measured.

Wettability

Wettability of the membranes was estimated by water drop shape analysis (DSA 10, Krüss, Germany). Water contact angle was averaged from 10 droplets with a volume of 0.20 µl which were placed on both surfaces of the membranes.

Tensile test

Tensile testing was conducted with universal testing machine (Zwick 1435, Germany). The test speed was 100 mm/min with 0.1 N pre-load, specimen length of 40 mm and specimen width of 5 mm

Percentage of PEG leached out

To ensure PEG removal, leaching out was performed by excessive washing with UHQ water. Percentage of PEG leached out from the membrane was calculated from the equation (1):

$$PEG\_leach\_out(\%) = 100\% - \frac{W_0}{W_f} * 100\% \qquad (1)$$

where W_0 and W_f were the weights of membranes before and after leaching, respectively.

Thickness

The thickness of each membrane was measured in six different places using a micrometer screw.

Biological experiment

Cell culture and seeding

Osteoblast-like cells MG-63 (LGC/ATCC, UK) were cultured in MEM (PAA, Austria) medium supplemented with foetal bovine serum (10%) (Sigma, Germany), penicillin-streptomycin (1%), sodium pyruvate (0.1%) and amino acids (0.1%) (PAA Austria). For subcultures, cells were trypsinized, suspended in culture medium and $1.3x10^4$ cells were seeded on each sample and cultured in 5% CO_2 atmosphere at 37°C. The cells were then analysed in two time points: 24 h and 6 days.

Cells morphology and distribution

The cells were fixed in paraformaldehyde and stained with acridine orange solution (1 mg/ml, Sigma, Germany) in order to perform a microscopic observation by fluorescence microscope (Zeiss Axiovert 40, Carl Zeiss, Germany).

Crystal violet

Crystal violet (CV) is a useful assay to analyze quantitatively the relative density of cells adhering to the material. First paraformaldehyde was used to fix the cells. Next the samples were washed with PBS and 0.5 ml of CV solution (0.5% CV in 20% of methanol) (Sigma, Germany) was added. After washing the samples with tap water, CV was extracted in 1 ml of 100% methanol. 10 minutes later, 100 µl of the supernatant were transferred to a 96 well-plate and the absorbance was measured at 570 nm using a Multiscan FC Microplate Photometer (Thermo Scientific, USA).

Protein content

To determine the total content of protein in the cells' supernatants, the bicinchoninic acid test (BCA, Sigma Germany) was carried out. The BCA reagent was prepared just before the assay by mixing $CuSO_4$ solution (4%) with BCA in the proportion of 1:50. Then 10 µl of the supernatant and 200 µl of BCA reagent were added to a 96 well-plate. After 30 min incubation in dark the absorbance was measured at 540 nm.

Nitric oxide level

Nitrite/nitrate production, an indicator of nitric oxide (NO) synthesis, was measured in cell culture supernatants by the Griess reaction. To perform this assay Griess reagent A – 0.1% naphthalethylenediamine dihydrochloride (Sigma Germany) in water and Griess reagent B – 1% sulfanilamide (POCH Poland) in 5% H_3PO_4 were mixed (1:1, v/v). Next 100 µl of the supernatant and 100 µl of the Griess reagent (A+B) were transferred to 96-well plate. The absorbance was measured at 540 nm.

Results

Characterization of PLGA membranes

Atomic force microscopy

FIG. 1 shows AFM pictures for scan areas of 20 µm x 20 µm and 100 µm x 100 µm of both sides (top, e.g. air-cured and bottom, e.g. glass-cured) of the membranes produced with two PEGs differing in molecular weight (200 Da and 1450 Da). It can be seen that PLGA/PEG 200 has similar porous topography on both sides. The size of pores (TABLE 1) and average roughness (FIG. 2) of this membrane are the same. On the other hand PLGA/PEG 1450 membrane has an asymmetric microstructure: the top surface is non-porous, while the bottom surface has the highest roughness and the biggest pores.

Wettability

The contact angle measurements were carried out to evaluate wettability of PLGA/PEG membranes. The results are presented in TABLE 1. The contact angle is about 85° for all the surfaces, except for bottom surface of PLGA/PEG1450, for which contact angle is about 10 degrees lower. However the results are not significantly different according to t-test ($p < 0.05$).

Tensile test

Mechanical properties of the samples were evaluated in tensile test and three parameters were measured: tensile strength Rm (MPa), Young's Modulus (MPa) and total elongation at break ε_{Ftotal} (%). TABLE 2 shows the results for the membranes and FIG. 3 shows representative stress-strain curves. Both membranes have similar tensile strength and Young's modulus but PLGA/PEG 200 membrane has significantly higher elongation at break.

PEG leached out percentage and membranes' thickness

The results of percentage of PEG leached out and membranes' thickness are shown in TABLE 2. As it can be seen PEG leaching out from both membranes is slightly higher than 60 wt%. The thickness of the membranes, as expected, is about 50 µm.

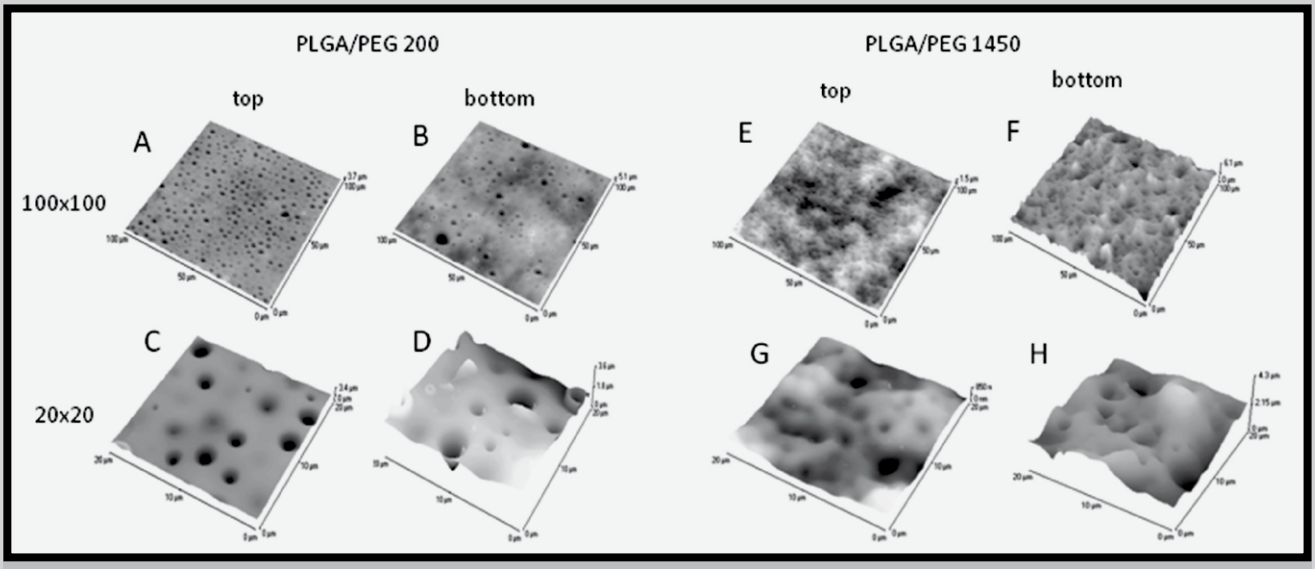

FIG. 1. AFM images of PLGA membranes obtained with use of PEG 200 (A,B,C,D) and PEG 1450 (E,F,G,H); top surface (A,C,E,G), bottom surface (B,D,F,H); scanned areas: 100 µm x 100 µm (A,B,E,F) and 20 µm x 20 µm (C,D,G,H).

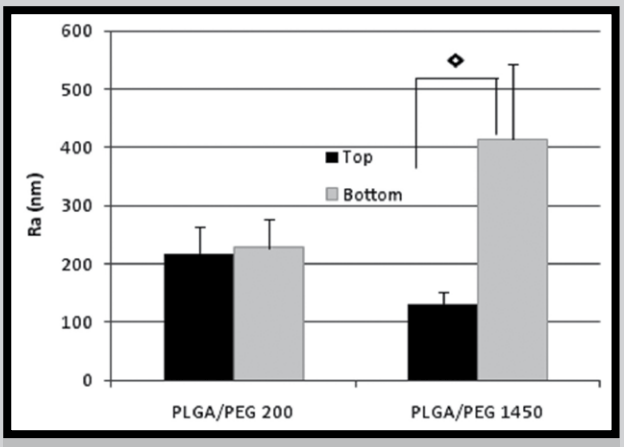

FIG. 2. Roughness obtained by AFM image analysis for top and bottom surfaces of the membranes: PLGA/PEG 200 and PLGA/PEG 1450. Data are expressed as average ± standard deviation, n=3. Diamond (♦) indicates statistically significant difference according to t-test between the groups: p<0.05.

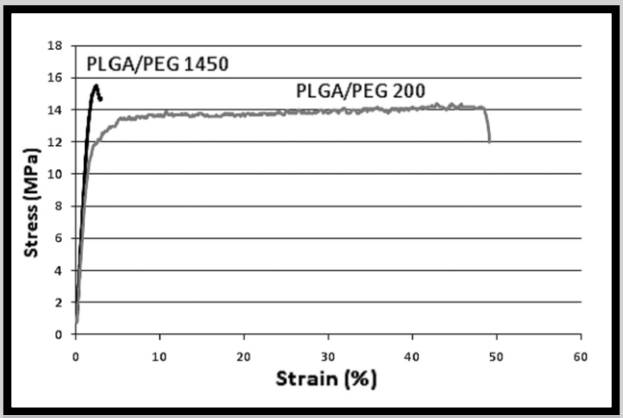

FIG. 3. Tensile test representative curves of PLGA membranes obtained using different molecular weight of PEG.

TABLE 1. Pore size and contact angle of PLGA/PEG membranes obtained with different molecular weight of PEG. Data are expressed as average ± standard deviation, n=30-40 for pore size and n=10 for contact angle, nd=not detectable. Diamond (♦) indicates statistically significant difference according to t-test between the groups: p<0.05.

Material		Pore Size [µm]	Contact Angle [θ]
PLGA/ PEG 200	Top	3.30 ± 0.18	89 ± 6
	Bottom	3.75 ± 0.13	83 ± 6
PLGA/ PEG 1450	Top	nd	85 ± 5
	Bottom	5.75 ± 0.20♦	76 ± 15

TABLE 2. Mechanical properties, thickness and percentage of PEG leached out of PLGA membranes (PLGA/PEG) obtained with different molecular weight of PEG. Tensile strenght (Rm), Young's modulus (E-Modulus), total elongation at break (ε_{Ftotal}). Data are expressed as average ± standard deviation, n=6 for thickness, n=3 for PEG leached out and n=5 for tensile test. Diamond (♦) indicates statistically significant difference according to t-test between the groups: p<0.001.

Membrane	Rm [MPa]	E-Modulus [MPa]	ε_{Ftotal} [%]	PEG leached out [wt%]	Thickness [µm]
PLGA/ PEG 200	13.8 ± 1.1	724 ± 16	49 ± 18♦	63.3 ± 0.7	50 ± 0.7
PLGA/ PEG 1450	13.3 ± 3.1	750 ± 141	4.7 ± 2.6	62.8 ± 0.4	51.5 ± 4.9

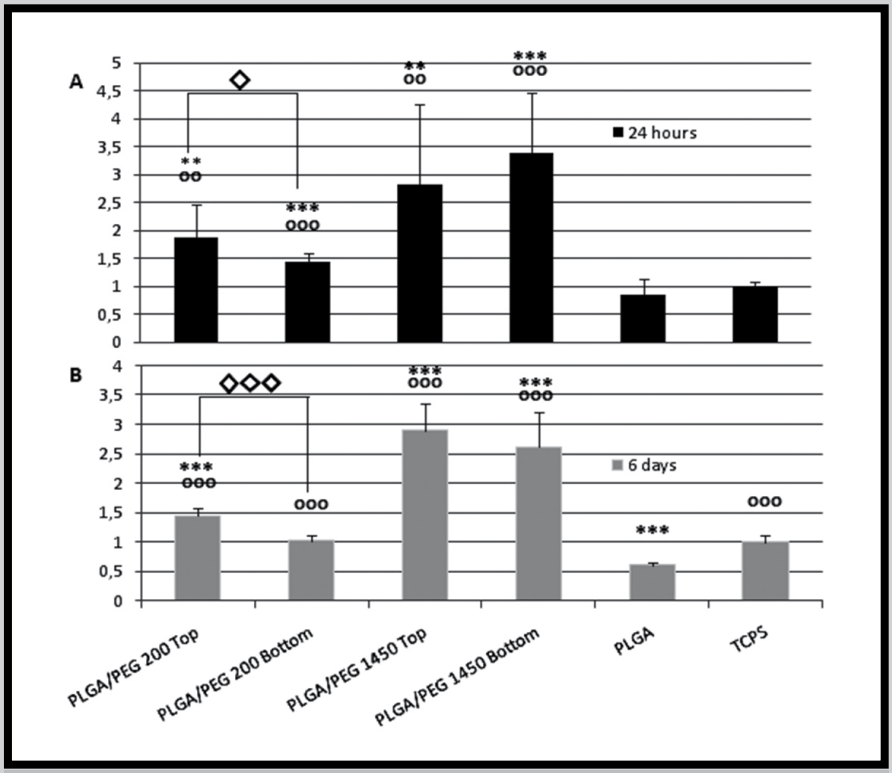

FIG. 4. Measurement of cells' attachment by crystal violet test performed after 24 h (A) and 6 days of culture (B) on PLGA/PEG 200 and PLGA/PEG 1450 membranes of both surfaces (top and bottom), PLGA foil and TCPS as the control (total TCPS absorbance = 1). Data are expressed as average ± standard deviation, n=4. Asterisks (*), circles (o) and diamonds (♦) indicate statistically significant differences from the TCPS control, PLGA and between the groups, respectively: *p<0.05; **p<0.01; ***p<0.001.

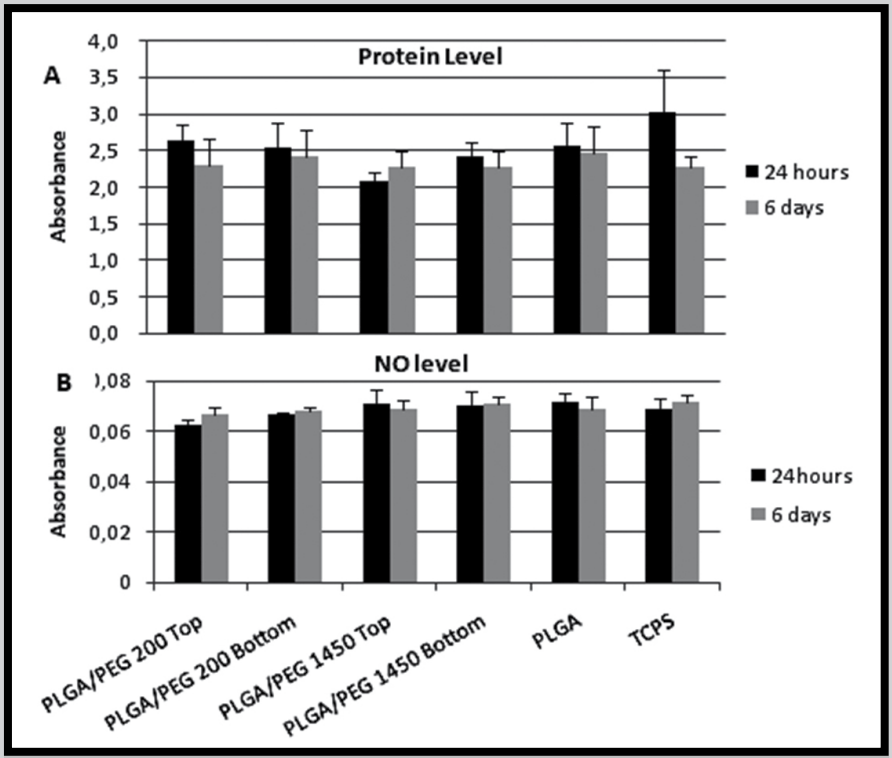

FIG. 5. Level of proteins (A) and level of nitric oxide NO (B) detected in the supernatants from cell cultured for 24 h and 6 days on PLGA/PEG 200 and PLGA/PEG 1450 both surfaces, PLGA foil and TCPS. Data are expressed as average ± standard deviation, n=4. No significant differences were found according to t-test.

***In vitro* experiment**

Cells morphology and distribution

Fluorescence microscopy observation showed that the number of cells after 6 days was higher than after 24 h and the cells were well spread. On all the surfaces including control PLGA foil and TCPS the cells were distributed homogenously, except the bottom surface of PLGA/PEG 1450, where the cells tended to grow in agglomerates (data not presented).

Crystal violet

CV staining, which is often used for the indirect quantification of number of adherent cells, stains DNA that can be quantified in a spectrophotometer [20]. All results were recalculated in relation to the values obtained from control TCPS wells (total TCPS absorbance = 1). As it can be seen in FIG. 4 there are significant differences between control samples (TCPS and PLGA foil) and the membranes. The number of adherent cells is higher on the membranes than on controls after 1 day. The same tendency is visible after 6 days. However, after 6 days of culture, there is also a significant difference between PLGA and TCPS. Moreover, significant differences between both surfaces of the membrane PLGA/PEG 200 are found.

Protein content

The BCA protein assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cation Cu^{1+} by bicinchoninic acid [21]. The obtained results from this experiment are presented in FIG. 5A. The total amount of protein in the supernatants from cell cultures on all the materials is similar in both time points. There are not statistically significant differences between the samples.

Nitric oxide level

NO plays an important role in several physiological processes including vascular regulation, immune responses and neural communication [22]. The NO value is similar for both time points and materials evaluated (FIG. 5B).

Discussion

The main purpose of this work was to produce resorbable membranes by phase separation/porogen leaching and evaluate their physical, mechanical and biological properties. Two different types of PLGA membranes were produced with PEG 200 Da and 1450 Da, as pore formers. Surface and mechanical properties of the membranes were characterized. Moreover the membranes were tested in contact with osteoblast-like cells to study their cytocompatibility.

The total percentage of PEG leached out from the blends (TABLE 2) of around 63%, was slightly higher than the expected value, because the ratio PEG/PLGA used to produce the membranes was 60/40. One can assume that excessive washing was enough to ensure the PEG removal, creating a porous structure; however it probably accelerated hydrolytic degradation of the PLGA structure.

In order to study the membranes' morphology, AFM analysis was carried out. There was a considerable difference in the membranes' topography: PLGA/PEG 200 top and bottom surfaces presented similar Ra values and similar size of pores while PLGA/PEG 1450 had distinct values for each surface. The air-cured surface was non-porous, while on the glass-cured surface bigger pores were present.

This fact can be explained by the formation mechanism of the porous structure. Due to higher molecular weight of PEG 1450, PEG-rich domains were bigger and sediment to the bottom surface; that is why, after leaching, this surface was more porous than the air-cured surface [23]. On the other hand the use of PEG with low molecular weight led to a homogeneous distribution of PEG-rich domains in PLGA-rich phase. For these reasons PLGA/PEG 200 membrane was more homogeneous while PLGA/PEG 1450 membrane presented an asymmetric microstructure. The results from this study are consistent with our previous results for PLGA membranes obtained with the use of PEGs with molecular weight in the range 300 – 3400 Da [24].

The wettability test showed that for more smooth surfaces, the contact angle was higher, meaning the surface was more hydrophobic. This is in agreement with Wentzel theory, which is describing the influence of the surface roughness on wettability [25]. Another possible explanation of lower contact angle on bottom surface of PLGA/PEG 1450 membrane is that hydrophilic PEG was not leached out properly. However this hypothesis should be ruled out, because percentage of PEG leached out from the membrane was as expected.

The mechanical analysis by the tensile test confirmed that application of PEGs with different molecular weight resulted in the membranes with different mechanical characteristics. The membranes had the same tensile strength and Young's modulus, but different elongation at break (FIG. 3, TABLE 2). The higher flexibility of PLGA/PEG 200 membrane is an important parameter for material handling during the surgery.

Biological tests were performed in order to evaluate the properties of the materials important from the point of view of possible medical applications. With the aim to verify cell morphology and distribution during cell culture, a fluorescent microscopy assay was done. The cells adhered and grew on the membranes with a cell number increasing from day 1 to day 6. Microscopic observations showed that higher roughness and porosity of the bottom surface of PLGA/PEG 1450 membrane on day 1 resulted in agglomerated groups of cells within the pores which are not well spread and therefore the material was not homogeneously colonized. After 6 days the cells colonized the entire surfaces of the membranes.

Crystal violet staining was conducted in order to study the cells attached after 24 h and 6 days of culture. The results show a high absorbance value for the PLGA/PEG 1450 membrane in both time points and surfaces (FIG. 4). However these values are not credible because probably the dye remained trapped in the membrane especially in PLGA/PEG1450 and therefore it is rather difficult to infer about the adhered cell number from these data.

The results of protein level (FIG. 5A) demonstrated the same protein concentration after 24 h than 6 days of culture. This can be explained by the fact that in the beginning of cell culture the medium was fresh, i.e. proteins from serum were present in the medium. At day 6 probably an equilibrium between consumed and produced proteins was reached.

It is well known that NO plays an important role in cellular metabolism. Although a high value of NO can involve toxicity including disruption of mitochondrial respiration, enzyme inhibition, lipid peroxidation and genetic mutation. This toxicity is mediated by intermediates such as N_2O_3 and peroxynitrite [26]. As it was verified (FIG. 5B) the NO level was low for the cells cultured on all the materials suggesting that the materials are not toxic for osteoblast-like cells.

Conclusions

It was demonstrated in this study that solvent-casting leaching-out PEG from PLGA/PEG blends is a very useful method to obtain PLGA porous membranes prospective for biomedical applications. Mechanical properties, microstructure, pore size and distribution of the pores in the membranes can be controlled by molecular weight of PEG used. Interestingly, higher molecular weight PEG provides asymmetric membranes with non-porous skin in the air-cured surface and the membranes are rather brittle. Contrarily, low molecular weight PEG provides membranes with homogenously distributed pores within the membranes, which are highly deformable. It was proven that PLGA membranes allow cellular colonization *in vitro*, which is of key importance for GTR application in the clinic.

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